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Somatic deletions of genes regulating MSH2 protein stability cause DNA mismatch repair deficiency and drug resistance in human leukemia cells

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Abstract

DNA mismatch repair enzymes (e.g., MSH2) maintain genomic integrity, and their deficiency predisposes to several human cancers and to drug resistance. We found that leukemia cells from a substantial proportion of patients (~11%) with newly diagnosed acute lymphoblastic leukemia (ALL) have low or undetectable MSH2 protein levels (MSH2-L), despite abundant wild-type *MSH2* mRNA. MSH2-L leukemia cells contained partial or complete somatic deletions of 1–4 genes that regulate MSH2 degradation (*FRAP1*, *HERC1*, *PRKCZ*, *PIK3C2B*); these deletions were also found in adult ALL (16%) and sporadic colorectal cancer (13.5%). Knockdown of these genes in human leukemia cells recapitulated the MSH2 protein deficiency by enhancing MSH2-

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degradation, leading to significant reduction in DNA mismatch repair (MMR) and increased resistance to thiopurines. These findings reveal a previously unrecognized mechanism whereby somatic deletions of genes regulating MSH2 degradation result in undetectable levels of MSH2 protein in leukemia cells, MMR deficiency and drug resistance.

In humans, DNA mismatches are recognized by one of two heterodimers, both of which contain MSH2: hMutSα (MSH2–MSH6) preferentially recognizes and repairs base-base mismatches as well as small insertion and deletion loops, whereas hMutSβ (MSH2–MSH3) recognizes and repairs small insertion and deletion loops. DNA polymorphisms (SNPs) and somatic mutations in *MSH2* are associated with DNA repair deficiency, leading to genomic instability and a higher risk of certain cancers (e.g., colon cancer [HNPCC], brain tumors, leukemia, lymphoma)[1, 2]. DNA mismatch repair (MMR) deficiency can also alter the sensitivity of cancer cells to thiopurine chemotherapy, both *in vivo* and *in vitro*[3, 4, 5]. All previously reported mechanisms of MSH2 deficiency directly involve the *MSH2* gene via mutations[6, 7, 8], LOH[9], or promoter methylation[10].

Although ALL is now curable in over 80% of children, the cause for treatment failure in the remaining patients remains unclear. We and others have reported that primary leukemia cells from a subset of patients with either ALL or acute myeloid leukemia (AML) have low levels of MSH2 protein, by mechanisms that have been hitherto unknown[4, 11, 12].

We initially measured MSH2 protein and mRNA expression in leukemia cells isolated from diagnostic bone marrow aspirates of 90 children with newly diagnosed ALL. MSH2 protein levels varied > 10-fold (range, < 6 – 102.9 RU%; mean ± SD, 36.0 ± 19.8 RU% [RU, relative unit]); notably ALL cells from ten patients (11.1%) had very low MSH2 protein (MSH2-L, < 2ng/10^6 cells or < 6–8 RU%) (Fig. 1a and Supplemental Fig. 1a). MSH2-L cases also had low levels of MSH6 protein, as expected in the absence of MSH2 (Supplementary Fig. 1b). We also confirmed the low level of MSH2 protein by immunohistochemistry (data not shown).

MSH2 mRNA was expressed at similar levels in ALL cells with low and high MSH2 protein (*n = 90*, *P = 0.63*, Fig. 1b). This was confirmed by quantitative real-time PCR analysis of cells from patients with MSH2-L ALL (*n = 7*) and MSH2-H ALL (*n = 10*) (data not shown). Moreover, we found no correlation between *MSH2* mRNA expression and MSH2 protein expression among ALL cells with high MSH2 protein (correlation coefficients r = 0.1, *P = 0.4*). There were no single-nucleotide polymorphisms (SNPs) in *MSH2* that differed in frequency between the two MSH2 phenotypes, nor were somatic mutations found in the *MSH2* mRNA of MSH2-L patients. Taken together, this pointed to a post-transcriptional mechanism for the observed MSH2 protein deficiency, consistent with prior studies showing a lack of correlation between MSH2 protein levels and *MSH2* mRNA expression in acute myelogenous leukemia cells from adult patients or in drug-selected cancer cell lines[12, 13, 14, 15]. Mechanisms responsible for this discordance in *MSH2* mRNA and protein levels in cancer cells are unknown, although mutations in the 3′ UTR of *MSH2* can create or destroy miRNA binding sites, thereby causing translational inhibition[16]. Indeed, we found one patient with MSH2-deficient leukemia cells who was heterozygous for an *MSH2* 3′ UTR SNP (2846T>G; rs17225053) that creates such a miRNA target site, consistent with

the reported 2% allele frequency of this SNP. However, given the low frequency of this SNP and its absence in other MSH2-L cases, it did not explain the MSH2-L phenotype we found in 11% of patients.

Because our prior studies revealed short chromosomal deletions (< 1 Mb in size) as the predominant copy number alteration in ALL[17], we examined DNA from leukemia cells in all cases with sufficient DNA for analysis (7 MSH2-L and 62 MSH2-H) using Affymetrix 6.0 SNP arrays to identify copy number changes in our candidate genes of interest, with an average resolution of approximately 5 kb[17]. No copy number changes were found in or around the MSH2 coding region in any cases. Because absence of PKCZ leads to enhanced degradation of MSH2[18], we initially looked for deletions in *PRKCZ* in cases with MSH2 protein deficiency, revealing deletions in 3 of 7 MSH2-L cases compared to only 1 of 62 MSH2-H cases (adjusted $P = 0.0066$). We then expanded this analysis to interrogate seven additional genes in the pathway upstream of *PRKCZ* (Fig. 1c; *AKT, PI3K2CB, PP2A, FRAP1, HERC1, TSC1, TSC2*). Based on chromosomal losses covering at least two consecutive SNPs, *FRAP1* deletions were found in 5 of 7 MSH2-L patients versus 2 of 62 MSH2-H cases (adjusted $P = 0.00062$), *HERC1* deletions were found in 4 of 7 MSH2-L cases versus 2 of 62 MSH2-H cases (adjusted $P = 0.020$) and *PIK3C2B* deletions were found in 2 of 7 MSH2-L cases versus 0 of 62 MSH2-H cases (adjusted $P = 0.091$). Collectively, all MSH2-L cases had deletions of at least one of these four genes, with 4 having deletions in two or more genes (Fig. 1d and Supplemental Fig. 2). Five of 62 MSH2-H cases had deletions in one of these genes, but none of the MSH2-H cases had deletions in more than one of these genes (Fig. 1d). Thus, penetrance of the MSH2-L phenotype was ~100% (95% CI = 40 –100%) when two or more of these genes had deletions, compared to ~58% (95% CI = 30–86%) when one or more of these genes had deletions. The frequency of deletions in the other genes in this pathway (*TSC1*, *TSC2*, *AKT1*, *PP2A*) was not significantly different in MSH2-L and MSH2-H cases.

The deletions found in MSH2-L cases were confirmed by quantitative PCR in all 6 cases with additional DNA for analysis (Supplementary Table 1). Sufficient leukemia cells to perform western blot analysis of deleted genes was available for two cases with hemizygous deletions of *FRAP1* and two cases with hemizygous deletions of *PRKCZ*, documenting ~30-60% lower amounts of the corresponding proteins in patients with deletions compared to cases without these deletions (matched for ALL lineage) (Fig. 2a**)**.

In an independent cohort of 170 ALL cases, 21 had evidence of deletions of one or more of these 4 genes (12.3%), a frequency comparable to the discovery cohort. Western blot analysis of leukemia cells from the validation cohort (i.e. cases with sufficient cells for analysis) revealed that 6 of 7 cases with deletions of one or more of these four genes had low MSH2 protein levels, whereas none of 14 controls (matched for ALL lineage and molecular subtype) without these deletions had low MSH2 protein levels (Fig. 1e and Supplemental Fig. 1c). The one discordant patient with a high level of MSH2 protein had a deletion of only one gene (*HERC1*), whereas all patients with deletions of two or more genes had low MSH2 protein. These data thus fully validated findings in the discovery cohort.

We assessed the microsatellite instability (MSI) status of DNA in primary ALL cells from patients with the MSH2-L (*n = 6*) and MSH2-H phenotypes (*n = 5* matched for ALL subtype) (Supplementary Table 2), revealing a higher number of markers showing instability in MSH2-L cases (Supplementary Fig. 3 and Supplementary Table 3).

Among the 252 patients with ALL treated according to the SJCRH Total Therapy XV protocol[19], we compared overall survival in the 97 patients for whom we measured MSH2 protein levels in their ALL cells (the overall treatment outcome of the 97 patients was comparable to the entire population of 252, data not shown). There was no difference between the MSH2-L and MSH2-H cohorts when compared for patient age, race, ALL genetic subtype, ALL lineage or patient sex (Supplementary Table5) and in the percentage of cells in S phase(Supplementary Fig. 6). As depicted in Figs 2b and 2c, patients whose ALL cells had low MSH2 protein levels (MSH2-L, *n = 16*) had a worse overall 10-year survival (78.7% ± 25.7% versus 97.5% ± 10.8%, respectively; *P = 0.009*, Log-rank test). Moreover, the 10 year cumulative incidence of hematological relapse was 20.6 ± 11.2% in the MSH2-L cases compared to 5.1 ± 2.5% in the MSH2-H ALL cases (*P = 0.06*, Gray test). In a multivariate analysis that included patient age, race, white blood cell count at diagnosis, ALL lineage (T or B) and the level of minimal residual disease (MRD) on day 19 of treatment (*n = 92* patients with all data), MSH2 phenotype remained significantly related to overall survival (*P = 0.032*, hazard ratio 17 [1.3-231]). The MMR system mediates the cytotoxicity of some DNA-damaging anticancer agents, and the absence of MSH2 has been shown to increase resistance to thiopurines[4, 5, 20]. We determined the sensitivity of human leukemia cells (CEM) to thiopurines (thioguanine or mercaptopurine) and other antileukemic agents, after individual knockdown of each of these genes. The IC50s for thioguanine and mercaptopurine (Fig. 2d and Supplementary Fig. 4 and Table 4) were significantly higher in leukemia cells in which *PIK3C2B*, *HERC1*, *FRAP1* or *PRKCZ* had been knocked down, as previously shown for MSH2 deficiency, whereas these cells were not more resistant to melphalan, daunorubicin, asparaginase, vincristine or glucocorticoids (Fig. 2d and Supplementary Fig. 4). Primary ALL cells from patients with somatic deletion of one or more of these four genes also exhibit greater resistant to mercaptopurine (Supplementary Fig. 5)

As shown in Fig. 3a–d, the individual knockdown of each of these genes resulted in a significant reduction in the level of MSH2 protein (67% to 49% reduction compared to controls, *P < 0.007*). This effect was confirmed by using a second independent shRNA against each of these genes (Supplementary Fig. 7). Each of these knockdowns also leads to low MSH6 protein levels (Fig. 3a–d), as anticipated in the absence of MSH2[21]. The effect of FRAP1 inhibition on MSH2 protein levels was further confirmed by the dose-dependent effects of rapamycin on MSH2 protein levels (Fig. 3e). A comparable effect of rapamycin on MSH2 was also observed in the human 697 B-lineage ALL cell line (Supplementary Fig. 8). As shown in Supplementary Fig. 9, when we simultaneously knocked down *PRKCZ* and inhibited FRAP1 with rapamycin, there was an even greater reduction in MSH2 protein levels.

In the control cells after cycloheximide treatment, we found endogenous MSH2 protein to be quite stable with very little degradation over 48 hours (Fig 3g–h). After knockdown of

PIK3C2B, *HERC1*, *FRAP1* or *PRKCZ*, there was more rapid loss of MSH2 protein (Fig 3g–h). There was increased ubiquitination of MSH2 after inhibition of FRAP1, and the more rapid degradation of MSH2 was blocked by the proteasome inhibitor MG132 (Fig. 3i–j and Supplementary Fig. 10).

To further elucidate the mechanisms by which these genes influence MSH2 protein levels, we assessed the effects of *PIK3C2B*, *HERC1*, and *FRAP1* knockdown on PP2A phosphatase activity. FRAP1 is known to inhibit the activation of PP2A, a critical regulator of PKCζ[22, 23]. HERC1 is a ubiquitin ligase that destabilizes TSC2[24], and TSC2 in complex with TSC1 inhibits FRAP1 function[25, 26]. PI3KC2β, belongs to the class II PI3Ks, but little is known about the physiological function of this class[27]. After knockdown of *PIK3C2B*, *HERC1* or *FRAP1*, there was a 2–3 fold increase in PP2A phosphatase activity compared to the control ($P < 0.01$) (Fig. 4a). We further showed that knockdown of *PIK3C2B* decreased the phosphorylation of AKT and P70S6K1 (also known as S6K1) at serine 473 and threonine 389, respectively (Fig. 3c). Likewise, knockdown of *HERC1* or *FRAP1* decreased P70S6K1 phosphorylation, consistent with mediation of their effects via FRAP1. There was also a dose-dependent effect of rapamycin, resulting in a 3 fold increase of PP2A activity at the highest concentration (Fig. 4a). As shown in Figs 4b and 4c, knockdown of *FRAP1*, *HERC1* or *PIK3C2B* led to a reduction in PKCZ phosphorylation (activation), an anticipated consequence of increased PP2A activity. To determine whether the decrease in MSH2 protein levels is via modulation of PP2A activity, we assessed the effects of the phosphatase inhibitor okadaic acid (10 nM). As shown in Figs 4b and 4c, okadaic acid treatment increased PKCζ phosphorylation (i.e., PKCZ activation) and restored MSH2 protein levels in cells in which either *HERC1, FRAP1* or *PIK3C2B* had been knocked down.

To assess functional consequences of *HERC1*, *FRAP1*, PIK3C2B or *PRKCZ* knockdowns, we measured DNA mismatch repair activity using nuclear extracts from CEM cells after knockdown of *HERC1*, *FRAP1*, *PIK3C2B* or *PRKCZ a*nd from NALM6 cells as an MSH2-deficient control. We observed that knockdown of *FRAP1*, *HERC1*, *PIK3C2B* or *PRKCZ* produced approximately 50% reduction in MSH2 protein, leading to a significant reduction in DNA mismatch repair capacity in human leukemia cells (Fig. 4d). These results are consistent with a haploinsufficiency model for MSH2's effects on DNA mismatch repair, as previously reported[28, 29, 30]. This reduction in MMR capacity was rescued by adding 0.2μg of purified MutSα to the repair reactions (Fig. 4d). The decrease in MMR function was comparable to the reduction in MMR observed following partial immune-depletion of MSH2 (Figs 4e and 4f).

To determine whether deletion of these four genes occurs in other human cancers, we analyzed publicly available datasets for sporadic colorectal cancer and adult ALL. In the colorectal dataset[31] 14 of 104 cases (13.5%) had deletions of one or more of these 4 genes (Supplementary Table 6). In the adult ALL cohort[32], deletions of one or more of these 4 genes were detected in 7 of 45 cases (16%) (Supplementary Table 7). About 15% of sporadic colorectal cancers have been reported to have a high level of microsatellite instability. Hypermethylation of the *MLH1* promoter region frequently causes inactivation of this gene, but this does not explain all sporadic colon cancer cases with the MSI-H phenotype. Prior work has shown that MSH2 protein expression was absent in ~15% of

sporadic colon cancer (i.e., 7 of 46 cases) and of these 7 cases, only 2 had LOH or somatic mutation of *MSH2*[33]. For the majority of these colon cancer cases (5 of 7) with low MSH2 protein (~11% of all cases), the mechanism leading to low MSH2 protein was not identified. It is plausible that these cases may be caused by deletions of genes regulating MSH2 protein stability.

In conclusion, inactivation of DNA mismatch repair genes is known to be involved in the pathobiology of certain hereditary and sporadic cancers, and has been associated with mutator phenotypes, inhibition of apoptosis, defective cell cycle arrest and chemotherapy resistance[34, 35, 36]. However, neither the genomic cause nor the functional consequences of MSH2-deficiency in primary leukemia cells has been previously established. The current work has identified a new genomic mechanism by which the leukemia cells of approximately 11% of patients with newly diagnosed ALL acquire MSH2 deficiency with multiple downstream consequences, and we found evidence that these same somatic deletions occur in sporadic colon cancer and other human malignancies.

Methods

Patients with ALL (initial discovery and validation cohorts)

We initially studied 90 patients (age ≤ 21 years) who had newly diagnosed ALL and were enrolled on the St. Jude Total Therapy XV protocol. To validate our findings, we subsequently studied an additional 170 patients with ALL enrolled on the same treatment protocol, using publicly available SNP data we had previously reported[17]. The treatment and research protocol was approved by the Institutional Review Board of St. Jude Children's Research Hospital, and informed consent was obtained from patients, their guardians, or both before enrollment. Patient assent was also obtained from patients who were 14 years or older. The diagnosis of ALL was based on previously described morphological and molecular criteria. Leukemia cells were isolated by applying a Ficoll-Hypaque gradient to bone marrow aspirates obtained at diagnosis (median, 97% blast cells). Normal leukocytes were isolated from peripheral blood samples obtained after the successful completion of remission induction therapy (on days 45–48 after the start of treatment).

Affymetrix 600K SNP array analyses

To assess gene copy number loss in MSH2-L leukemia cells, DNA was extracted from leukemia cells and normal peripheral blood leukocytes (obtained when patients were in complete remission) and genotyped for 600K SNPs using the Affymetrix GeneChip Human Mapping 50K-Hind-240, 50K-Xba-240, 250K-Sty and 250K Nsp SNP arrays. DNA was restriction enzyme digested, PCR-amplified, purified, labeled, fragmented and hybridized to the arrays according to the manufacturer's instructions. SNP array data were analyzed using dChip for chromosomal abnormalities. To improve the accuracy of copy number inference using dChip, we applied a normalization procedure that uses SNPs exclusively from regions shown to be diploid in the normal leukocytes and maps signals from those SNPs to a common target probability distribution. The SNPs with an estimated copy number lower than 1.40 were considered as evidence of deletions[17].

We initially performed SNP analysis on *PRKCZ*, because it is known to regulate MSH2 stability. This led us to subsequently perform a broader pathway analysis, to interrogate additional genes upstream of *PRKCZ* (Figure 1c). This included eight genes and a total of 122 SNPs. For each SNP, we evaluated the over-representation of deletions in MSH2-L leukemia cells compared to MSH-H leukemias, using Fisher's Exact test. The significance of each SNP was then adjusted for multiple testing using 100,000 permutations. At each permutation, we recorded the smallest p-value among all the 122 SNPs based on randomly assigned MSH2 status. The adjusted p-values were computed as the proportion of permutations whose smallest p-values were lower than or equal to the observed.

Cell culture

The human T-lineage leukemia cell line CCRF-CEM was obtained from the American Type Culture Collection. The human pre-B leukemia cell lines 697 and NALM-6 were obtained from the German Collection of Microorganisms and Cell Cultures. Cells were cultured in RPMI-1640 medium containing 2 mM glutamine and 10% fetal bovine serum at 37 °C with 5% CO_2.

Stable Short Hairpin RNA (shRNA) knockdowns

CCRF-CEM cells were infected with MISSION lentiviral transduction particles (Sigma-Aldrich) produced from a library of sequence-verified shRNAs targeting human *PIK3C2B*, *FRAP1/MTOR*, *HERC1* or *PRKCZ* transcripts. Non-target shRNA control particles (SHC002V) were also purchased from Sigma-Aldrich. Individual cell clones were isolated in medium containing puromycin.

Statistical analyses

Fisher's exact test was used to test the overrepresentation of losses among MSH2 low patients versus MSH2 positive patients. Fisher's exact test was performed at a single SNP level. A linear regression model was used to test the correlation between the levels of MSH2 mRNA and protein. The expression levels of the probe sets were analyzed by applying a general linear model in which the effect of MSH2 status (positive vs. negative) was adjusted for the ALL genetic subtypes. Overall survival rates were compared with the stratified Mantel-Haenszel test. The Cox proportional hazards model was used to identify independent prognostic effect of MSH2 status. For patients who achieved complete remission, cumulative incidence of hematological relapse were constructed by the method of Kalbfleisch and Prentice, and compared with Gray's test. All other failures were considered competing events. Fine and Gray's model was used to identify independent prognostic factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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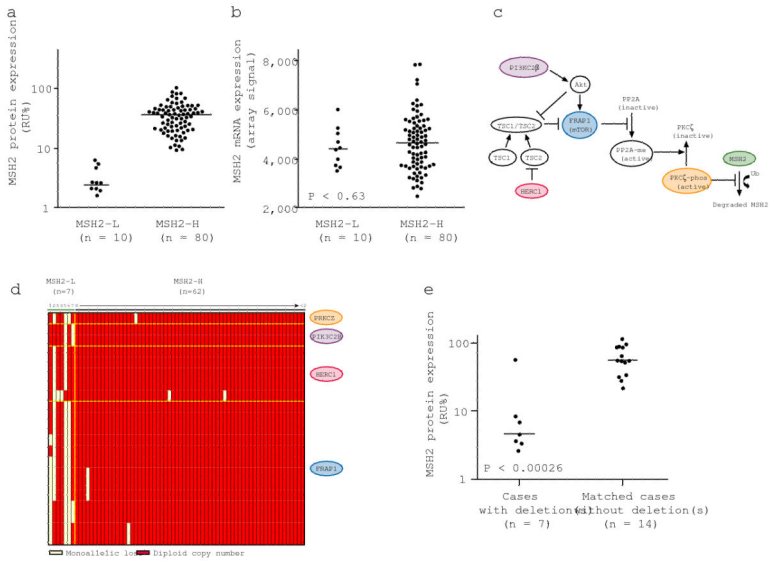

Figure 1.

Gene copy number loss and MSH2 protein expression in primary human leukemia cells. MSH2 protein (**a**) and mRNA (**b**) expression measured in primary acute lymphoblastic leukemia (ALL) cells isolated from bone marrow aspirates of 90 newly diagnosed patients with ALL constituting the discovery cohort. MSH2 protein levels (**a**) were determined by Western blot analysis and normalized to the GAPDH signal. MSH2-L was defined as cells with low MSH2 protein signals on a western blot of 1 million ALL cells (<10% RU). *MSH2* mRNA expression (**b**) was determined by Affymetrix gene expression array. Differences in the *MSH2* mRNA or protein levels were assessed using the exact Wilcoxon-Mann-Whitney test. The closed circles indicate values in individual cases. (**c**) Pathway of genes upstream of PKCζ and MSH2 degradation. Depicted in color are four genes that were deleted significantly more frequently in MSH2-L leukemias. (**d**) SNP array data were analyzed using dChip, revealing over-representation of mono-allelic copy number loss (yellow) in MSH2-L leukemia cells. (**e**) MSH2 protein levels in leukemia cells from the validation cohort (i.e., all cases with deletions of one or more of these genes with sufficient cells for western analysis, and a 2:1 matched cohort of ALL cases without these deletions). Quantification of the MSH2 protein levels normalized to the GAPDH signal in the validation cohort documents a significant difference between cases with deletions compared to those without deletions of the four genes in their ALL cells (*P = 0.00026*). Horizontal lines in panels a, b and e depict median values for each cohort.

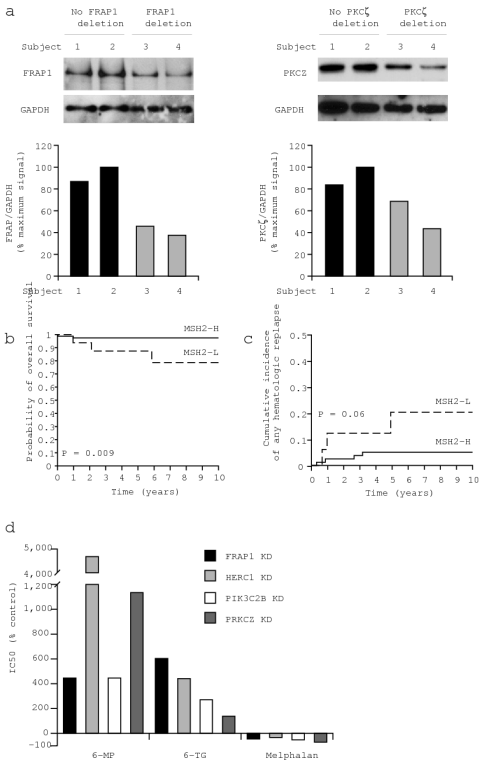

Figure 2.

Protein expression in primary leukemia cells with hemizygous deletions, treatment outcome and drug sensitivity according to leukemia cell MSH2 phenotype. (**a**) Western blot analysis of two cases with hemizygous deletions of *FRAP1* and two cases with hemizygous deletions of *PRKCZ* show ~30–60% lower amounts of the corresponding protein compared to the matched cases that did not have these deletions. Kaplan-Meier analysis of overall survival (**b**) and cumulative incidence of hematological relapse (**c**) in children whose ALL cells had the MSH2-L phenotype compared to those with the MSH2-H phenotype. The difference in overall 10 year survival is statistically significant (*P = 0.009*, Log-rank Test), whereas the difference in cumulative incidence of a hematological relapse approached the conventional level of statistical significance (*P = 0.06*, Gray test). (**d**) CCRF-CEM cells in which *PIK3C2B*, *HERC1*, *FRAP1* or *PRKCZ* were knocked-down exhibit significantly greater resistance to the thiopurines antileukemic agents 6-thioguanine (6-TG) and 6-mercaptopurine (6-MP). In contrast these cells exhibited increased sensitivity to the alkylating agent melphalan. Cells were treated for 72 hours in the presence of increasing concentrations of 6-TG, 6-MP or melphalan. Cell viability was determined by the MTT assay.

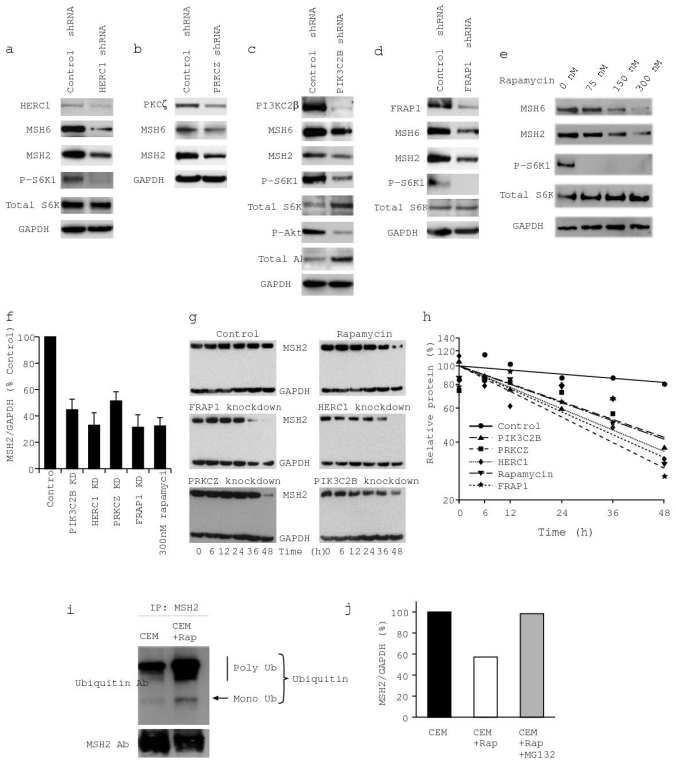

Figure 3.

PRKCZ, *PIK3C2B*, *HERC1*, *FRAP1* inhibition and MSH2 stability. Human leukemia cells (CCRF-CEM) were transduced with shRNA against *PRKCZ*, *PIK3C2B*, *HERC1*, *FRAP1* or with the non-target control. Western blot analysis was used to determine the effect of these knockdowns on each protein knocked down and on MSH2 protein levels after *HERC1* (**a**) *PRKCZ* (**b**) *PIK3C2B* (**c**) and *FRAP1* (**d**) stable knockdown. (**c**) Phosphorylation of AKT (Ser 473) and P70S6 kinase (Thr389) were measured by phospho-specific antibodies in panels a, c, d, e. (**e**) Depicts the decrease in MSH2 protein levels following treatment with increasing concentrations of rapamycin to inhibit FRAP1. (**f**) MSH2 protein levels after *PRKCZ*, *PIK3C2B*, *HERC1*, or *FRAP1* inhibition. MSH2 protein levels were quantified by densitometry, normalized to GAPDH signal and expressed as a percent of the control. Values are means ± SD of three independent experiments. MSH2 protein levels were significantly lower ($P < 0.007$) after each knockdown or after treatment with rapamycin (300 nM), when compared to controls. (**g**) To determine the half-life of MSH2 protein, CCRF-CEM cells were treated with 5μg ml^{-1} cycloheximide to inhibit protein synthesis. Leukemia cells were harvested at indicated time points in SDS cell lysis buffer before being fractionated in 4–12% Nupage gels and immunoblotted with MSH2 and GAPDH antibodies. (**h**) The level of MSH2 protein on Western blots was quantified by densitometry and normalized to the GAPDH protein signal. The results expressed as a percent of the time zero value show a shorter half-life of MSH2 after *PRKCZ*, *PIK3C2B*, *HERC1*, *FRAP1* inhibition. (**i**) Inhibition of FRAP1 by rapamycin (300 nM) resulted in accumulation of ubiquitinated MSH2 protein in CEM cells after 48 hours and (**j**) the lower MSH2 protein levels were reversed by the proteasome inhibitor MG132 (10 nM).

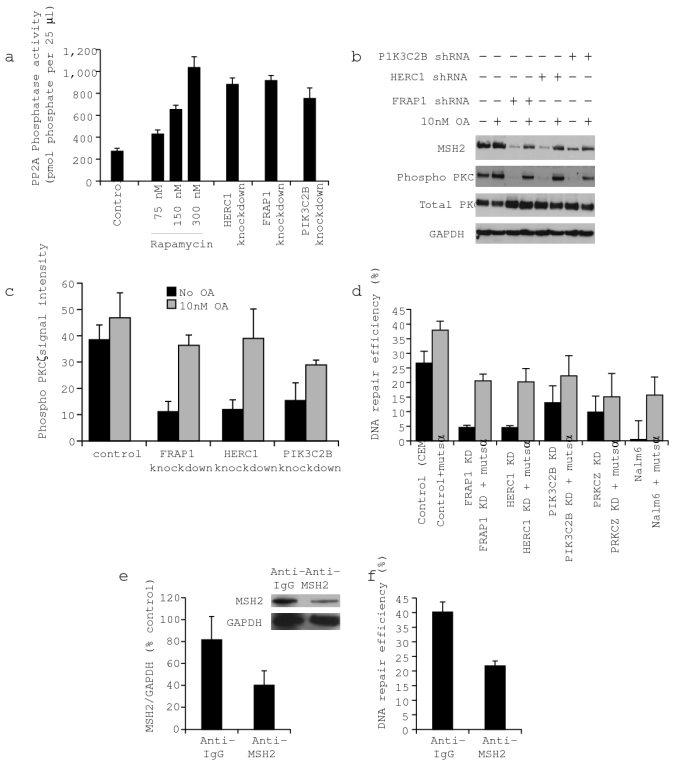

Figure 4.

Increase in PP2A activity via inhibition or knockdown of *FRAP1*, *HERC1 or PIK3C2B* with rescue by okadaic acid (OA) and effects on MMR activity. (**a**) Phosphatase activity was assayed colorimetrically using threonine phosphopeptide, K-R-pT-I-R-R, as a substrate. Error bars represent standard deviations of three replicate experiments. PP2A activity was significantly higher than controls after each knockdown or rapamycin treatment ($P < 0.01$). (**b**) Increase of PP2A activity by *FRAP1*, *HERC1* or *PIK3C2B* knockdown decreases PKCzeta activation (phosphorylation) and MSH2 protein levels, both of which are rescued by okadaic acid. OA+ indicates that cells were incubated 24h with 10 nM of okadaic acid. Whole cell extracts were resolved by SDS-PAGE and immunoblotted for MSH2, GAPDH, PKCζ and the active form of PKCζ (phosphorylation of threonine 410). (**c**) Phospho PKCζ signal was quantified by densitometry. Values are means ± SD of three independent experiments. (**d**) Using a *M13mp2* DNA substrate containing a two base loop, the repair efficiency is expressed as a percentage: 100 × (ratio of mixed colonies in the control group – the ratio of mixed colonies in repaired group)/(ratio of mixed colonies in the control group). Each experiment was performed in triplicate, and data represent the mean ± S.D. of three separate determinations. DNA repair efficiency was significantly lower than controls after each knockdown ($P < 0.02$) and this effect was rescued by the addition of purified hMutSα. (**e**) Cytosolic extracts were depleted of MSH2 with antibody or were mock-depleted with mouse IgG. The data are averages from three (mock-depleted, MSH2-depleted) experiments. *Error bars* represent the mean ± S. D. of three independent experiments (**f**) MSH2 immuno-depletion decreases MMR activity. Mock-depleted and MSH2-depleted cytosolic extracts

were used to determine MMR activity as described above. DNA repair efficiency was significantly lower after MSH2 depletion (*P = 0.004*).